Summary of the Ph.D. Thesis

Macro-organization and Structural Flexibility of the Photosynthetic Pigment System in Diatoms

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INTRODUCTION

During photosynthesis, plants, algae and certain bacteria transform the photon energy of the sunlight to chemical energy, which is essential in the synthesis of biomolecules necessary for energy storage in the metabolic processes. Diatoms (Bacillariophyta) are unicellular eukaryotic photosynthesizing algae and they play essential role in global primary production and in the regulation of atmospheric CO_2 concentration.

The components of the photosynthetic electron transport chain are highly conserved in eukaryotes, therefore they are quite similar in higher plants and diatoms. However, the organization of thylakoid membranes and pigment-protein complexes of diatoms displays several differences compared to higher plants; the thylakoid membranes are not differentiated into granum and stroma lamellae and they are arranged into groups of three stacked membranes, which span the whole length of the chloroplasts.

In higher plants, the photosystem II (PSII) and its accessory light-harvesting complex (LHCII) is located in the stacked granal thylakoid membranes, while photosystem I (PSI) with its accessory light-harvesting antenna (LHCI) can be found in the non-stacked stroma thylakoids. In contrast, in diatoms the localization of PSII and PSI in the membrane is homogeneous, no lateral heterogeneity could be observed. The main light-harvesting complexes of diatoms are the fucoxanthin-chlorophyll protein (FCP) complexes, which serve as accessory antennae for both PSI and PSII. In diatoms, the arrangement of PSII, PSI and FCP complexes are homogeneous, no lateral heterogeneity could be observed.

It is well known in higher plants that PSII-LHCII supercomplexes are arranged into chirally ordered macrodomains, as it has been shown by circular dichroism (CD) spectroscopy (Garab and Amerongen 2009). CD spectroscopy is a powerful, non-invasive method for the investigation of structural properties of complex biological systems and to follow the structural changes. In hierarchically organized systems the CD signals originate from different molecular structures. In the case of chlorophyll molecules weak intrinsic CD signals can be observed, which originate from the inherent asymmetry and chirality. In the case of interactions of two or more pigment molecules (which occurs e.g. in isolated pigment-protein complexes) excitonic CD signals can be observed, which are characterized by conservative band structure. In large, chirally ordered macroaggregates

anomalous CD signals with large intensity and non-conservative band structure can be observed, which can be associated with differential light-scattering. These CD bands are called as polymer or salt-induced (psi) type signals.

In higher plants, the chirally organized macrodomains exhibit a remarkable structural flexibility. It has been shown that they are capable of undergoing temperature and light-induced structural reorganizations as indicated by the large variations in the intensity of the psi-type CD signals, while the excitonic interactions were much more stable. It has also been established that the osmotic pressure of the medium and divalent cations are essential in maintaining the chiral macroorganization of the chromophores.

Our knowledge about the supramolecular organization of the pigment-protein complexes in diatoms is rudimentary, and the macro-organization of pigment molecules has never been investigated.

AIMS

In order to understand the photosynthetic light-harvesting processes, it is important to reveal the macro-organization of the pigment molecules in addition to the biochemical characterization of the components of the photosynthetic electrontransport chain. Therefore, the following aims were addressed in my PhD work:

I. To conduct systematic study on the macro-organization of pigments in different levels of structural complexity, on free pigment molecules, on isolated pigment-protein complexes and thylakoid membranes, and on intact cells of the diatoms *Phaeodactylum tricornutum* and *Cyclotella meneghiniana*

II. To investigate the effect of different environmental factors - temperature, strong illumination, light intensity during growth, osmotic pressure and ionic composition of the medium - on the chiral macrodomains and to correlate these possible changes with physiological parameters.

III. To investigate the microenvironment, the interactions and the heterogeneity of fucoxanthin (Fx), the most important light-harvesting carotenoid of diatoms.

METHODS

Culturing the diatom species

The diatoms *Phaeodactylum tricornutum* and *Cyclotella meneghiniana* were grown in ASP-2 medium. Cells were grown on a photon flux density of 40 μmol photons m^{-2} s^{-1} photosynthetically active radiation in a dark/light period of 16 h/8 h on 19 °C. For certain experiments cells were also grown on either low light (LL, 10-15 μmol photons m^{-2} s^{-1}) or high light (HL, 180-200 μmol photons m^{-2} s^{-1}).

Isolation of thylakoid membranes and pigment-protein complexes

Cells were disrupted in a French pressure cell. The thylakoids were isolated by differential centrifugation. The thylakoid membranes were solubilized in n-dodecyl β-D-maltoside and the pigment-protein complexes were separated by using sucrose density gradient ultracentrifugation.

Breaking up the intact cells by using ultrasound

Sonication of intact cells was performed in a Branson Sonifier 450 on ice in ASP-2 medium.

Determination of the chlorophyll content

The chlorophyll content was determined spectrophotometrically in acetonic pigment-extracts according to Jeffrey and Humprey (1975).

Absorption spectroscopy

Absorbance spectra of intact cells were recorded with a Shimadzu UV-3000 spectrophotometer, in split beam mode, in the wavelength range of 400 – 750 nm. In order to minimize the spectral artifacts caused by light scattering of turbid cell suspensions, the spectra were measured in a sample holder designed correcting for scattering.

Circular dichroism spectroscopy

CD spectra were measured with a Jobin-Yvon CD6 or a Jasco J-815 dichrograph in the wavelength range of 400-750 nm. The measurements of temperature-dependent CD-changes were performed in a thermostated sample holder. For kinetical measurements of the light-induced CD changes, illumination was provided with a side-illumination attachment. The CD spectra are plotted in absorbance units.

Linear dichroism spectroscopy

LD spectra were measured at room temperature in a Jobin-Yvon CD6 dichrograph, equipped with LD boards, in the wavelength range of 400-730 nm. Cells or thylakoid membranes were oriented with the gel-squeezing method or with a strong magnetic field. The LD spectra were measured in absorbance units. The orientation angles were calculated according to Garab (1996).

Fluorescence spectroscopy

The 77K fluorescence spectra were recorded with a Horiba Jobin-Yvon Fluorolog 3 spectrofluorimeter. Fluorescence emission spectra were measured between 600 and 800 nm, using 510 and 550 nm excitation wavelengths. The fluorescence excitation spectra were recorded between 400 and 600 nm; the emissions were measured at 689 or 713 nm.

Measurement of electrochromic absorbance changes

Flash-induced electrochromic absorbance changes were measured with a home-built single beam spectrophotometer according to Barabás et al. (1985) and Büchel and Garab (1995). To determine the transient spectra of the electrochromic absorbance changes, kinetic traces were measured between 470 and 570 nm. The electrochromic signals are expressed in $-\Delta I/I$ units. In order to reveal the absorbance bands of the electrochromically shifted pigments, the transient spectra were fitted with the first derivatives of Gaussians.

Measurement of the chlorophyll fluorescence transients and the determination of the photosynthetic parameters

Room temperature fluorescence measurements were performed using a PAM 101 Chl fluorometer (Walz, Effeltrich). The maximal quantum efficiency of PSII photochemistry of thylakoids in reaction buffers was calculated as F_v/F_m, where F_v and F_m are called as variable and maximum fluorescence, respectively. NPQ was calculated as NPQ = F_m/F_m'-1, according to Bilger and Björkmann, 1990.

Determination of the pigment composition by high performance liquid chromatography (HPLC)

Samples were resuspended in HPLC medium (90% methanol/0.2 M ammonium acetate (90/10, v/v) and 10% ethylacetate). Pigments were analyzed on a Waters 600 MS chromatography system An ET 250/4 Nucleosil 300-5 C18 column was used for separation. Eluents and gradient programs were as described in Kraay et al. 1992.

Transmission electron microscopy

Cells were visualized by Zeiss 902 electron microscope. The samples were pelleted by centrifugation. The pellet was fixed with phosphate-buffered glutaraldehyde. Samples were postfixed with phosphate-buffered osmium-tetroxide, gradually dehydrated in ethanol, incubated in propylene oxide and then hardened in the resin Araldite. Ultrathin sections were cut with ultramicrotome. Sections were taken up onto copper grids. For contrasting the specimen, sections were stained with uranyl acetate and lead citrate.

SUMMARY OF THE RESULTS

I. In the diatom *Phaeodactylum tricornutum* weak intrinsic CD signals could be detected in extracted pigment molecules. Isolated thylakoid membranes exhibited CD bandpair at (+)445/(-)478 nm, which is an excitonic CD signal and originates mainly from fucoxanthin (Fx). At 679 nm a negative band could be observed which cannot be considered as excitonic band, because it does not exhibit conservative band structure. Intact cells exhibit large CD bands at around 698 nm. This band is associated with

differential scattering and disappears when cells are disrupted, thus it can be considered as a psi-type CD band originating from chiral macrodomain organization of the chromophores.

By using sucrose gradient centrifugation, it was possible to separate the trimeric and oligomeric FCP complexes (FCP and FCPo, respectively). The CD spectra of FCP and FCPo are very similar to each other, and psi-type CD signals could be observed in neither of them, thus FCP complexes themselves do not form chiral macrodomains.

The psi-type property of the (+)698 nm band could be verified by using sonication of the cells. Upon breaking up the cells, the intensity of the (+)698 nm band decreases and finally disappears, while the (-)679 nm band changes only to smaller extent. The transmission electron microscopic pictures of control and sonicated cells confirmed that the psi-type band exists only if the thylakoid membranes are arranged into multilamellar system.

II. The chiral macrodomains exhibited remarkable flexibility upon changing different environmental factors in *P. tricornutum* cells. By using consecutive heat treatment on intact cells, the psi-type band proved to be much more sensitive than the excitonic bands; the intensity of the psi-type CD signal decreased already at lower temperatures, while the excitonic bands remained essentially unchanged up to 45 °C. Above this temperature the excitonic CD signals disappeared, indicating the disassembly of pigment-protein complexes.

Light-induced CD changes could also be observed in intact cells; the psi-type band and – to lesser extent – the (-)679 nm band displayed changes, while the other spectral changes remained essentially unchanged. The light-induced CD changes saturated in 100 s and they were reversible as it has been shown by kinetic measurements The magnitude of the light-induced CD changes depended on the applied light intensity up to about 450 μmol photons m^{-2} s^{-1}, at which they become saturated. The xanthophyll cycle inhibitor dithiothreitol inhibited the light-induced CD changes, which suggests the involvement of xanthophyll cycle in the structural changes. However, the proton gradient uncoupler ammonium chloride did not affect significantly the light-induced CD changes in *P. tricornutum*, therefore at present it is not clear how the NPQ and the changes in the

macrodomain order are correlated; further experiments are needed to clarify the relationship of the structural changes and NPQ in diatoms.

Intact cells grown on different light conditions displayed remarkably different CD spectra. Cell grown on low light (LL) exhibited much larger psi-type signal and weaker signal at (-)679 than cells grown on high light (HL). In contrast, the kinetics and amplitude of the light-induced CD changes were much smaller than in HL cells. The presence of the large psi-type signal accompanied by a smaller flexibility may be advantageous for the enhanced light-harvesting capability, which is crucial in the adaptation to lower light intensities.

The increase in the ambient osmotic pressure caused reversible changes mainly in the psi-type CD signal, albeit the excitonic interactions were also affected to little extent. In isolated thylakoid membranes the psi-type CD signal disappeared. However, when cells were isolated in the presence of $MgCl_2$, the psi-type signal could be well retained. The psi-type signal could be partially restored when thylakoids (isolated in the absence of $MgCl_2$) were incubated in $MgCl_2$-containing buffers. Along with the psi-type signal, the functional parameters F_v/F_m and NPQ values and the deepoxidation ratio were also significantly higher in the presence of Mg^{2+} ions.

The chiral macro-organization of the pigments was also observed in another diatom species, *Cyclotella meneghiniana*. The psi-type band was identified at (+)694 nm. This band was sensitive to illumination with strong light and heat treatment, while the excitonic bands were unaffected by these treatments. In the presence of Mg^{2+} ions the psi-type CD band could be well retained in isolated thylakoid membranes.

III. In order to obtain information about the microenvironment of Fx molecules, flash-induced electrochromic absorbance changes were measured between 470 and 570 nm on intact *P. tricornutum* cells. Two main electrochromic signals were identified at 515/485 and 565/535 nm, originating from different spectral form of Fx molecules, and due to their wavelength positions they could be interpreted as the analogues of Fx_{red} and Fx_{green}, respectively, as it has been found earlier in isolated FCP complexes (Premvardhan et al. 2008). We found that different light conditions during growth affects the amount of the different Fx forms; LL grown cells accumulate Fx_{red} compared to HL grown cells, while the amount of Fx_{green} did not change considerably. Low temperature fluorescence

spectroscopy measurements revealed that Fx_{red} displays a somewhat more efficient excitation energy transfer to chlorophyll *a* emitting at 689 nm than Fx_{green}, which was true for both HL and LL cells. We have also established that Fx_{red} molecules possess smaller orientation angle respect to the membrane plane than Fx_{green}.

Similar electrochromic signals were identified in intact cell of another diatom species, *C. meneghiniana*. The Fx molecules are spectrally and functionally heterogeneous in this species as well and the Fx_{red} és Fx_{green} forms could also be identified. These results suggest that heterogeneity of Fx molecules can be a general feature in diatoms.

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